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14. ABSTRACT

We hypothesized that exposure to environmental factors could impact gene expression in the brain and could play a key role in environmental-induced cases of autism. Accordingly, we established mouse embryonic cortical cultures of neurons and astrocytes, and exposed them to commonly occurring epigenetic modifying agents. To study the effects of such exposure on the methylation status of neuronal and astrocytes DNA, we designed primers for bisulfite DNA sequencing and methylation specific PCR of CpG islands of autism-associated genes, Bdnf, Gad67, Mecp2, and Reln). To study the effects of environment exposure on gene expression, we used RT Real Time PCR. mRNA from primary neurons was reverse transcribed and used in the assays. DNA samples from primary neurons treated as reported was prepared and frozen. They will be analyzed by BS and MSP.

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Introduction

Autism is a heterogeneous neurodevelopmental disorder characterized by impaired social interaction and communication, and restricted and repetitive behavior. This disorder has a strong but unclear genetic component and is likely caused by the complex interplay of alleles of various sets of susceptibility genes. We proposed to investigate possible links between exposures to specific, relevant environmental agents/dietary factors and epigenetic changes in autism-related genes using embryonic mouse primary cortical neurons and in astrocytes. We hypothesized that such factors could profoundly impact gene expression in the brain during development and, in combination with undefined susceptibility factors, could play a key role in environmental-induced cases of autism. We proposed to study the effects of physiological concentrations of commonly occurring epigenetic modifying agents on the expression, and methylation status of autism-associated genes: Bdnf, Gad67 (Gad1), Mecp2, and Reln.

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To perform such studies, we used Methyl Primer Express to design primers for bisulfite DNA sequencing (BS) or methylation-specific PCR for either methylated CpG islands (MS) or unmethylated islands (NMS). The primers (Table 1, Table 2, and Table 3) allow to assess the methylation status of a) Gad67 (-1133 to –942 5' UTR; Dong et al., 2007); b) Reln (-414 to +242 5'UTR; Dong et al., 2007); c) Mecp2 (-618 to +1207); and d) Bdnf CpG islands associated with the exon IV promoter-enhancer region. Some of the primers were synthesized according to data published by others (Dennis and Levitt, 2005; Dong et al., 2007). Bisulfite treatment of DNA was done using a commercial available kit (Zymo Research). We tested the primers using bisulfite treated DNA from adult mouse brains. Figure 1 shows PCR products obtained with a number of primer sets for both bisulfite DNA sequencing and methylation specific PCR (Table 1, Table 2 and Table 3). The data indicated that the primers could be used for our proposed Methylation studies of primary embryonic cortical neurons and astrocytes.

To assess the possible links of exposure to environmental agents and methylation status of gene involved in autism, we used primary cortical neurons and astrocytes from BALB/c mice embryos at day 14. Upon arrival to our animal colony, female BALB/c mice received the Zeigler Phytoestrogen Reduced Rodent Diet II (Zeigler Brothers, Gardeners, PA) formulated for studies where dietary phytoestrogens, such as genistein can influence experimental results. Mice were maintained on the diet for the entire experiment.

Groups of 3 to 4 females were kept together with a male for mating (24 hours). After 14 days, pregnant mice were anesthetized with CO2 and sacrificed by cervical dislocation. The abdomen was wiped with 70% ethanol and cut to expose both horns of the uterus. Embryos were transferred to a Petri dish containing dissecting solution and decapitated. Under a dissecting microscope the skin and the skull was pulled away using two pairs of Dumont forceps. Holding the head with a pair of forceps such that the dorsal aspect of the brain was facing up, the meninges were pulled and the two hemispheres separated by passing a pair of forceps through the sagittal fontanelle. Cortices were flipped laterally and removed by pulling (movie). The isolated cortices were minced, trypsinized and mechanically triturated using Pasteur pipets to yield dissociated brain cells (Vicario-Abejon, 2004).

Table 1. Primers for Bisulfite Sequencing (BS), Methylation Specific (MS), and Non-Methylation Specific (NMS) PCR of CpG Islands of mouse Bdnf (exon IV).

Primer/Number	***Sequence 5'-3'***	***Assay***
BSeIV#1 F * 2	TAATGATAGGTTTGGTTTTGT	BS
BSeIV#1 R * 3	AAACTCCATTTAATCTAAACAAAA	BS
BSeIV#2 F 32	TGTTAGGATTGGAAGTGAAAAT	BS
BSeIV#2 R 4	AAACTCCATTTAATCTAAACAAAAACT	BS
BSeIV#8 F 1	AGGATTGGAAGTGAAAATATTTATAAAGTA	BS
BSeIV#8 R 29	AAAAACTCCATTTAATCTAAACAAAAACTA	BS
BSeIV#4 F 5	TGAATTTGTTAGGATTGGAAGTG	BS
BSeIV#4 R 31	GAAAACAATCCTCTCCTCGATAAA	BS
MeIV#1 F 15	TCGAGGAGAGGATTGTTTTC	MS
MeIV#1 R 24	AATAATACTCGCACGCCTTC	MS
NeIV#1 F 22	TTATTGAGGAGAGGATTCTTTTT	NMS
NeIV#1 R 10	AATAATACTCACACACCTTCAAC	NMS
MeIV#2 F 25	GGTAGCGTGGAGTTTTTTC	MS
MeIV#2 R 11	ATTTCATACTAACTCGCCGA	MS
NeIV#2 F 18	TAAGGTAGTGTGGAGTTTTTTT	NMS
NeIV#2 R 19	ATTTCATACTAACTCACCAAAAA	NMS
MeIV#3 F 21	AGAGGATTGTTTTCGTTGTGTC	MS
MeIV#3 R 7	CGAAAATAATACTCGCACG	MS
NeIV#3 F 6	AGGAGAGGATTGTTTTTGTTGTT	NMS
NeIV#3 R 20	CAAAAATAATACTCACACACCT	NMS

(*) Primers of each set: Forward (F) and Reverse (R) are listed next to each other.

Table 2. Primers for Bisulfite Sequencing (BS), Methylation Specific (MS), and Non-Methylation Specific (NMS) PCR of CpG Islands of mouse genes: Gad67 (gad), Reln (reln) and Mcp2 (mec).

Primer/Number	***Sequence 5'-3'***	***Assay***
Mgad F 16	GTTTTGTTTTTAAAGGATGAGAAAAC	MS
Mgad R 13	AAAAATTTCCACCAAAAAACG	MS
Ngad F 8	TTTGTTTTTAAAGGATGAGAAATCG	NMS
Ngad R 26	AAAATTTCCACCAAAAAACACC	NMS
Mreln F 28	GGGAGGGTTTTAGGATAAGC	MS
Mreln R 9	CCGAACTCATAACTACCCGTT	MS
Nreln F 30	GGAGGGTTTTAGGATAAGTGT	NMS
Nreln R 17	ACCCCAAACTCATAACTACCCAT	NMS
BSmcp#1 F 23	TTTGGTAGGAATAAGTTGAGGG	BS
BSmcp#1 R 27	TTTCAATAATTCAACCATAACCA	BS
BSmcp#2 F 14	TTTTTTGGGGATGTTGTGT	BS
BSmcp#2 R 12	ACCACTACCACATAAAAATCACC	BS

(*) Primers of each set: Forward (F) and Reverse (R) are listed next to each other.

Table 3. Primers for Bisulfite Sequencing (BS), Methylation Specific (MS), and Non-Methylation Specific (NMS) PCR of CpG Islands of Mcep2.

Primer	Sequence 5' – 3'	Assay
1MB F *	TTATTGGTTGTGGAGTTTAGGTT	BS
2MB R *	CAAACTCCTCAACAAACAACTT	BS
3M1 F	AGTAAGGGTTTTCGTTCGC	MS
4M1 R	TAATAAAACCCGTCCGAAA	MS
5N1 F	TTTAGTAAGGGTTTTTGTTTGT	NMS
6N1 R	CTATAATAAAACCCATCCAAAAA	NMS
7M2 F	TAATCGATGTCGGGATTTC	MS
8M2 R	AATTAAAAACGTCACCGCTAA	MS
9N2 F	GTAATTGATGTTGGGATTTT	NMS
10N2 R	CATTAAAAACATCACCACTAA	NMS
11M3 F	TGTGTTGTTGTATTTGCGC	MS
12M3 R	AAAGCTCACCGCTAAAACTC	MS
13N3 F	TTGTGTGTTGTTTCATTTGTGT	NMS
14N3 R	AAAAACATCACCACTAAAACTCC	NMS

(*) Primers of each set: Forward (F) and Reverse (R) are listed next to each other.

To prepare astrocytes, cells were resuspended in Dulbecco's modified Eagle medium supplemented with 10% FCS and plated on polylysine coated 6-well-plates (Falcon) at a density of $1.2x10^6$ cells per 3.5 cm well and cultured overnight in 5% CO2 at 37^o C. Medium was then replaced with Neurobasal™ (Invitrogen) supplemented with 1mM Glutamax (Invitrogen) and G-5 supplement (Invitrogen). For neuronal cultures, dissociated brain cells were resuspended in Nerobasal™ supplemented with 1mM Glutamax and B-27 supplement (Invitrogen), and plated on polylisine coated wells (Yamasaki et al., 2003). For this project, we used a total of 36 BALB/c mice. In average every week 2 females were 14 days pregnant and could be sacrificed for harvesting embryos (average 5 embryos per pregnant animal). Using the above protocols, we obtained cultures that contain prevalently neurons or astrocytes (Fig. 2). Next, we performed experiments to determine whether E14 cortical cells (primary neurons and astrocytes) growing in 35 mm dishes would yield sufficient DNA to perform methylation studies of all four genes. Cell lysates prepared either with DNeasy (Qiagen) or Trizol (Invitrogen) were used to make DNA or both RNA and DNA (Trizol). Bisulfite-treated DNA was then used in PCR assays with BS and methylation specific primers. As shown in Figure 3, the majority of primers worked well with DNA samples prepared with both reagents. Inasmuch as both RNA and DNA samples can be made from the same dish with Trizol, the results of the above experiments allowed us to cut in half the number of cell culture dishes necessary to perform both RNA expression and DNA methylation studies.

Embryonic cortical cells (E14) were processed (weekly) as described above and plated using the appropriated medium and growth factors. After 1-6 days incubation primary neurons and/or astrocytes were exposed (Tables 4– 15) to environmental agents (in triplicate, 35 mm dishes).

1 genistein a soy isoflavone that mimics the effects of estrogen in the body, (present in soy dietary and infant soy formula; induces gene methylation; Dolinoy et al., 2007);

2 fluoxetine (Prozac, a selective serotonin reuptake inhibitor); upregulates the expression of Mecp2 (Cassel et., 2006);

3 fluoxetine (BPA; induces gene demethylation; Dolinoy et al., 2007); found in plastic bottles and containers and detected as a toxic element in amniotic fluid and other human biological fluids;

4 Toll-like receptor (TLR) agonists for

- TLR2- tripalmitoylated lipopolipeptide (Pam3CSK4);
- TLR3- double stranded RNA (dsRNA; poly IC);
- TLR4- lipopolysaccharide (LPS);
- TLR7/8- GU-rich single-stranded RNA (ssRNA40);
- TLR9- unmethylated CpG-containing DNA (ODN 1826).

As controls, we analyzed untreated cells, and cells treated with valproic acid (VPA, 0.6-1.2 mM for 48 hr), 5-Aza-2'-deoxyctridine (5AzadC 1 uM for 48 hr), or trichostatin A (TSA, 333 μM for 48 hr). These drugs are histone deacetylase inhibitors that are known to induce CpG demethylation of Bdnf, Gad67 and Reln, which is associated with increased expression of the same genes (Kundakovic et., al 2008). After exposure cells, were lysed with Trizol.

Table 4. Primary embryonic neurons (144 hrs postplanting) exposed to different environmental agents. Cortical cells (10 E14) were plated (1.2 x 10^6 per dish) on 2/25/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (50 uM)	6	24	3/4/09
BPA control	6	24	3/4/09
Poly:IC (10ug/ml)	6	43	3/5/09
Poly:IC control	6	43	3/5/09
VPA (0.6 mM)	6	48	3/5/09
VPA control	6	48	3/5/09

Table 5. Primary embryonic neurons (120 hrs postplanting) exposed to different environmental agents. Brain cells (26 cortices E14) were plated (1.2 x 10^6 per dish) on 3/4/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (50 uM)	3	46	3/11/09
BPA control	3	46	3/11/09
Poly:IC (10ug/ml)	3	48	3/11./09
(20 uM)	3	46	3/11/09
VPA (0.6 mM)	3	46	3/11/09
CpG (10 ug/ml)	3	48	3/11/09
LPS (10 ug/ml)	3	48	3/11/09
Control *	3	48	3/11/09
TSA (333uM)	3	46	3/11/09
TSA control	3	46	3/11/09
Genistein (0.1 mM)	3	72	3/12/09
Genistein control	3	72	3/12/09

(*) the control was the same for Poly I:C, VPA, CpG, LPS, and Fluoxetine

Table 6. Primary embryonic neurons (120 hrs postplanting) exposed to different environmental agents. Brain cells (13 cortices E14) were plated (1.2 x 10^6 per dish) on 3/25/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (30 uM)	3	24	4/1/09
BPA control	3	24	4/1/09
CpG (10 ug/ml)	3	48	4/2/09
CpG Control	3	48	4/2/09
Fluoxetine (1 uM)	3	72	4/3/09
Genistein (10 uM)	3	72	4/3/09

Table 7. Primary embryonic astrocytes (120 hrs postplanting) exposed to different environmental agents. Brain cells (11 corticesE14) were plated (1.2 x 10^6 per dish) on 4/1/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (30 uM)	3	24	4/8/09
BPA control	3	24	4/8/09
CpG (10 ug/ml)	3	48	4/9/09
CpG Control	3	48	4/9/09
Fluoxetine (1 uM)	3	72	4/10/09
Genistein (10 uM)	3	72	4/10/09
Poly I:C (10 ug/ml)	3	48	4/9/09
VPA (0.6 mM)	3	48	4/9/09

Table 8. Primary embryonic neurons (120 hrs postplanting) exposed to different environmental agents. Brain cells (14 cortices E14) were plated (1.2 x 10^6 per dish) on 4/15/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
LPS (10 ug/ml)	3	48	4/23/09
Fluoxetine (1 uM)	3	48	4/23/09
TSA (333 uM)	3	48	4/23/09
Control *	3	48	4/23/09
Genistein (10 uM)	3	48	4/23/09
Genistein control	3	48	4/23/09

(*) the control was the same for TSA, LPS. and Fluoxetine

Table 9. Primary embryonic neurons (120 hrs postplanting) exposed to different environmental agents. Brain cells (14 cortices E14) were plated (1.2 x 10^6 per dish) on 4/22/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (10 uM)	3	24	4/29/09
BPA control	3	24	4/29/09
5AzadC (1 uM)	3	48	4/30/09
TSA (0.333 uM)	3	48	4/30/09
VPA (1.2 mM)	3	48	4/30/09
Control *	3	48	4/30/09

(*) the control was the same for 5AzadC, TSA, and VPA

Table 10. Primary embryonic neurons (120 hrs postplanting) exposed to different environmental agents. Brain cells (33 corticesE14) were plated (1.2 x 10^6 per dish) on 5/6/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
BPA (50 uM)	3	24	5/13/09
BPA control	3	24	5/13/09
Fluoxetine (1 uM)	3	24	5/13/09
Fluoxetine control	3	24	5/13/09
Poly I:C (10 ug/ml)	3	48	5/14/09
LPS (10 ug/ml)	3	48	5/14/09
CpG (10 ug/ml)	3	48	5/14/09
Pam3SK4 (10 ug/ml)	3	48	5/14/09
VPA (0.6 mM)	3	48	5/14/09
5AzadC (1 uM)	3	48	5/14/09
Control *	3	48	5/14/09
Genestein (5 uM)	3	48	5/14/09
Genestein control	3	48	5/14/09

(*) the control was the same for 5AzadC, Poly I:C, LPS, CpG, Pam3SK4, and VPA

Table 11. Primary embryonic neurons (48 hrs postplanting) exposed to different environmental agents. Brain cells (20 cortices E14) were plated (1.2 x 10^6 per dish) on 6/17/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
VPA (1.2 mM)	3	66	6/22/09
TSA (0.333 uM)	3	66	6/22/09
5AzadC (1 uM)	3	66	6/22/09
Poly I:C (10 ug/ml)	3	66	6/22/09
CpG (10 ug/ml)	3	66	6/22/09
Pam3SK4 (10 ug/ml)	3	66	6/22/09
Genistein (5 uM)	3	66	6/22/09
Control *	3	66	6/22/09

(*) the control was the same for all treatment

Table 12. Primary embryonic neurons (48 hrs postplanting) exposed to different environmental agents. Brain cells (16 cortices E14) were plated (1.2 x 10^6 per dish) on 6/24/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
VPA (1.2 mM)	3	72	6/29/09
Fluoxetine (1 uM)	3	72	6/29/09
LPS (10 ug/ml)	2	72	6/29/09
BPA (50 uM)	3	72	6/29/09
Control *	3	72	6/29/09

(*) the control was the same for all treatments

Table 13. Primary embryonic astrocytes (48 hrs postplanting) exposed to different environmental agents. Brain cells (16 cortices E14) were plated (1.2 x 10^6 per dish) on 6/24/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
VPA (1.2 mM)	3	72	6/29/09
Genistein (5 uM)	3	72	6/29/09
Poly I:C (10 ug/ml)	3	72	6/29/09
Pam3SK4 (10 ug/ml)	3	72	6/29/09
Control *	3	72	6/29/09

(*) the control was the same for all treatments

Table 14. Primary embryonic astrocytes (24 hrs postplanting) exposed to different environmental agents. Brain cells (13 cortices E14) were plated (1.2 x 10^6 per dish) on 7/1/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
VPA (1.2 mM)	3	24	7/3/09
Fluoxetine (1 uM)	3	24	7/3/09
BPA (50 mM)	3	24	7/3/09
Control *	3	72	6/29/09

(*) the control was the same for all treatments

Table 15. Primary embryonic astrocytes (48 hrs postplanting) exposed to different environmental agents. Brain cells (13 cortices E14) were plated (1.2 x 10^6 per dish) on 7/1/09.

Treatment (conc.)	*dish (number)*	*Exposure (hrs)*	*Harvest*
CpG (10 ug/ml)	3	72	7/6/09
LPS (10 ug/ml)	3	72	7/6/09
TSA (0.333 uM)	3	72	7/6/09
Control *	3	72	7/6/09

(*) the control was the same for all treatments

To study the effect of exposure to epigenetic agents on gene transcription, we used RT Real Time PCR. mRNA isolated from exposed primary neurons was reverse transcribed and used in the assays. Real Time PCR was performed using RT^2 qPCR SYBR-Green Assays (SABiosciences) with primers designed to target transcripts of autism-related genes: Bdnf , Gad67, Mecp2, and Reln. We analyzed mRNA samples from primary neurons treated with 5AzadC, TSA, and VPA (Table 16). The relative quantification of the expression of each target gene versus the expression of Hprt1 (reference gene) was calculated with the $2^{-\Delta\Delta C}{}_T$ Method (Livak and Schmittgen 2001) using the results from cultures exposed to epigenetic insults or not. Analysis of the data indicates that in the conditions used in the experiments there was a little increase in the expression of some of the genes. For example, treatment with 5AzadC (Figure 4, A) resulted in a 1.7 fold increase on Gad67 (Gad1) mRNA, and treatment with TSA (Figure 4,B), resulted in a 1.7 fold increase of Bdnf mRNA. However, the high standard deviation observed in some of the experiments shown in Figure 4, could be an indication of errors in handling the samples. DNA samples from primary neurons and/or astrocytes treated as reported (Table 10 to Table 16) were prepared and frozen. They will be analyzed by MSP and bisulfite DNA sequencing. In the latter method, DNA is extracted from cells exposed to environmental agents, treated with bisulfite and used in PCR with specific primers (Bdnf, Gad67, Mecp2 and Reln). The purified PCR products are then cloned and sequenced. DNA sequencing of a number of isolated clones (usually 10 –12) is necessary to assess the percentage of methylated cytosine at any given CpG site. Inasmuch as in our experiments (Table 5-Table 16), we have exposed neurons and astrocytes to numerous agents for various length of time, the assessment of the methylation status of CpG island has not yet be done. Moreover, we also intend to analyze CpG methylation by using mouse "Profiler DNA methylation PCR Arrays" (SABiosciences) which were custom made (SABiosciences) and tailored to detect simultaneously methylation of the CpG islands of six genes: Bdnf (3 sets of primers), Gad67, Mecp2 and Reln (Table 17). The arrays do not require DNA treatment with bisulfite and detect methylation with accuracy similar to that of bisulfite sequencing DNA sequencing. DNA samples to be analyzed are restricted either with an enzyme that requires the presence of methylated cytosine or one that works only in the absence of methylated cytosine. The relative amount of DNA remaining after each restriction respect to unrestricted and double restricted DNA is quantified by Real Time PCR using the standard ΔC_T method (data analysis software, SABiosciences). It is our hypothesis that dysregulation in gene expression will also be reflected by abnormal gene methylation.

Table 16. Quantitative PCR amplification of mouse transcripts using RT^{2}qPCR Primer Assay – SYBR Green Mouse (SABiosciences)

Gene	*Catalog #*	**RefSeq A #**	*Ref. Positions **	*PCR prod. (bp)*
Gad1 #	PPM36500E	NM_008077.4	1749-1769	86
Mecp2	PPM31215E	NM_010788.3	9593-9617	145
Reln	PPM24902E	NM_011261.2	11522-11542	175
Bdnf	PPM03006B	NM_007540.4	4671-3693	155
Hprt1	PPM03559E	NM_013556.2	883-902	83

(*) The sequence of the primers is proprietary and not available.
(#) Gad1 and Gad67 are used to indicate the same mouse gene

Table 17. Primers sets for Methyl-Profiler DNA Analysis (SABiosciences)

Gene	Symbol	Unigene #	Refseq#	Cat# of Primers
1	Gad1*	Mm.272120	NM_008077.4	MePM05982-3A
2	Mecp2	Mm. 131408	NM_010788.3	MePM11566-1A
3	Reln	Mm. 425236	NM_011261.2	MePM07987-1A
4	Bdnf	Mm. 1442	NM_007540-4	MePM06137-1A
5	Bdnf	Mm. 1442	NM_001048142.1	MePM06138-1A
6	Bdnf	Mm. 1442	NM_001048141.1	MePM06139-1A

(*) Gad1 and Gad67 are used to indicate the same mouse gene

Key Research Accomplishments

- We designed and synthesized set of primers for Methylation specific PCR and bisulfite sequencing.
- The above primers were capable to amplify the expected products.
- We established a system for mating: 3 to 4 females kept with a male for 24 hr, to obtain 14 days pregnant females.
- We were able to keep this system going for about 5 months and produce a steady supply of 14 days embryos.
- We established tissue cultures methods for the differentiation of embryonic cortical cell to primary neurons and astrocytes.
- We determined that neurons cultures contained a relative low number of astrocytes; and astrocytes cultures contained low number of neurons.
- We exposed both primary neurons and astrocytes to the chosen environmental agents
- We were able to reduce in half the number of dishes necessary for the numerous treatment, by demonstrating that RNA and DNA samples extracted from cells lysed with Trizol could be used for determining both mRNA levels (RealTime PCR) and CpG DNA methylation (BS, MSP).

Reportable Outcomes
We have not yet finished our experiments and we do not have reportable outcome.

Conclusion

To assess the possible links of exposure to environmental agents and methylation status of gene involved in autism, we used primary cortical neurons and astrocytes from BALB/c mice embryos. Groups of females (3–4 in a cage) were each mated weekly with a male for 24 hr to produce E14 embryos for the experiments. Cortical cells were plated and processed to obtain cultures that contain either prevalently neurons or astrocytes. We demonstrated that RNA and DNA samples extracted from cells (35 mm dishes) lysed with Trizol could be used for determining both mRNA levels (RealTime PCR) and CpG DNA methylation (BS, MSP). Cultures of primary neurons and/or astrocytes were exposed to genistein, fluoxetine, fluoxetine, and Toll-like receptor agonists: Pam3CSK4, poly IC, lipopolysaccharide, ssRNA40, and unmethylated CpG-containing DNA (ODN 1826). For each of the experiments, we processed the treated cells to prepare both RNA and DNA samples which were frozen for later analysis. We started our studies on the effect of exposure to epigenetic agents on gene transcription by using RT Real Time PCR. mRNA isolated from exposed primary neurons was reverse transcribed and used in the assays. Real Time PCR was performed using RT^2 qPCR SYBR-Green Assays with primers designed to target transcripts of autism-related genes: Bdnf , Gad67, Mecp2, and Reln. We analyzed mRNA samples from primary neurons treated with the control drugs 5AzadC, TSA, and VPA. It has been reported that the above agents induce CpG demethylation of Bdnf, Gad67 and Reln and result in increased expression of the same genes (Kundakovic et., al 2008). We found that in neurons treated with 5AzadC there was a 1.7 fold increase on Gad67 (Gad1) mRNA, and in neurons exposed to TSA, there was a 1.7 fold increase of Bdnf mRNA. There was no much variation in the expression of the other genes. It is possible that primary cortical cells at this stage of development (120 hr in tissue culture) are not very responsive to the agents at the dosages and length of exposure used in our protocol. Alternatively, the exposures to the agent could have induced epigenetic modifications of DNA, without triggering a concomitant

alteration of gene expression. Thus, it would of interest to study gene expression in cells kept in tissue culture for longer times and exposed to various dosages of agents. We still have to analyze the RNA and DNA samples obtained from our experiments. In our proposal, we underestimated the time necessary to establish: 1) mating groups of Balb/C mice for production of E14 embryos; 2) cell culture protocols for the preparation of primary neurons and astrocytes; 3) protocols for the analysis of gene expression and DNA methylation. We are confident that the analysis of the RNA and DNA samples will produce important data on the role of epigenetic insults as causative factors of autism.

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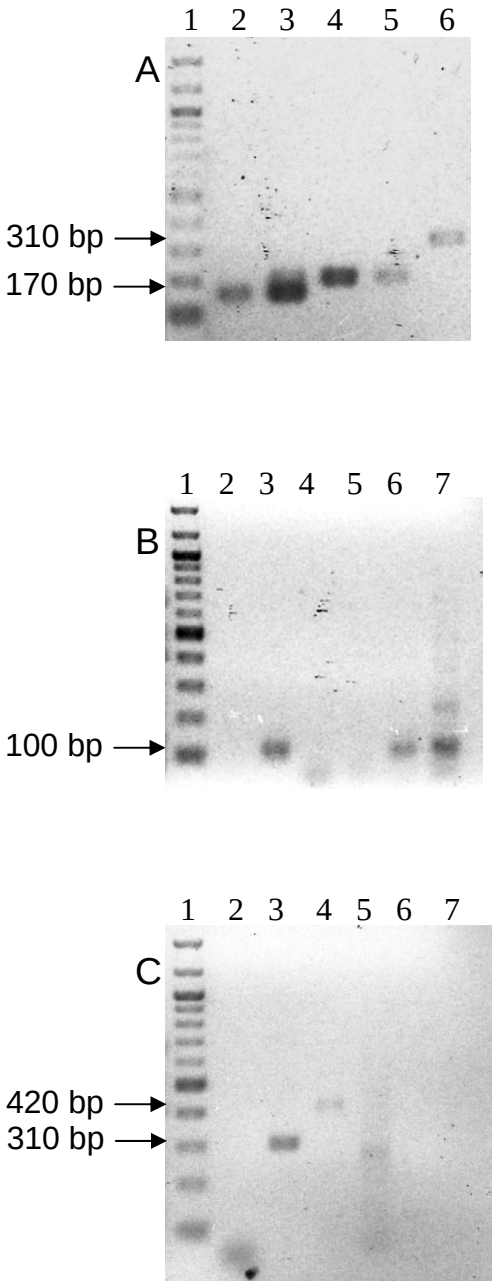
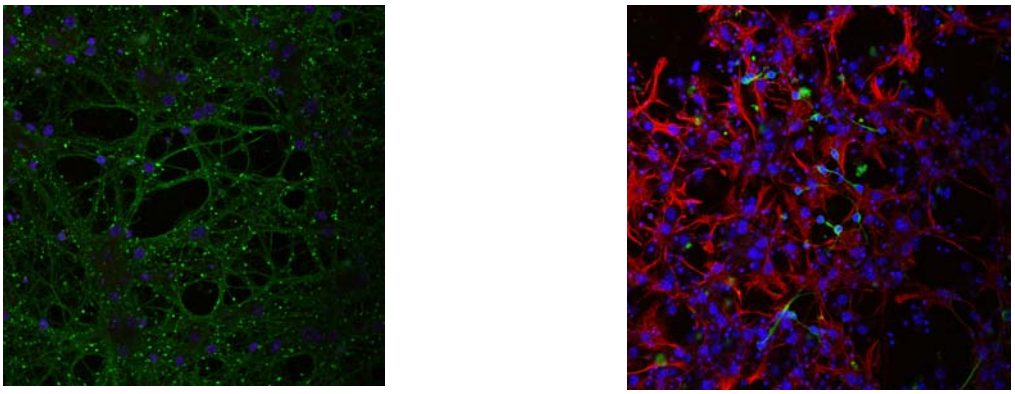


Fig. 1. PCR products obtained with bisulfite sequencing (BS) or Methylation Specific (MS, NMS) primers were analyzed by agarose gel electrophoresis. In lane 1 of each gel: 100 bp DNA ladder.
A: Reln primers F28/R9 (MS; lane 2) and F30/R17 (NMS; lane 3). Gad67 primers F16/MR13 (MS; lane 4) and F8/R26 (NMS; lane 5) (Table 2). Bdnf primers F5/R31 (BS; lane 6) (Table 1).
B: Bdnf primers (Table 1): F15/R24 (MS; lane 2); F22/R10 (NMS; lane 3); F25/R11 (MS; lane 4); F18//R19 (NMS; lane 5); F21//R7 (MS; lane 6); F6//R20 (NMS; lane 7).
C: Bdnf primers (Table 1): F2/R3 (BS; lane 2) and F32/R4 (BS; lane 3). Mecp2 primers: F23/R27 (BS; lane 4); F14/R12 (BS; lane 5).



A **B**

Fig. 2. Cortical cultures of neurons (A) in green and astrocytes (B) in red from BALB/c mouse embryos (E14), seeded onto coverslips and cultured for five days were fixed in 2% formaldehyde, permeabilized with 0.05% saponine and immunostained for neuronal and glia markers, using an anti-Beta tubulin mAb, and rabbit anti-GAFP antibody. As secondary antibodies we used anti-mouse-Alexa-488 and anti-rabbbit-Alexa-568. Nuclei were stained blue with TOPRO-3.

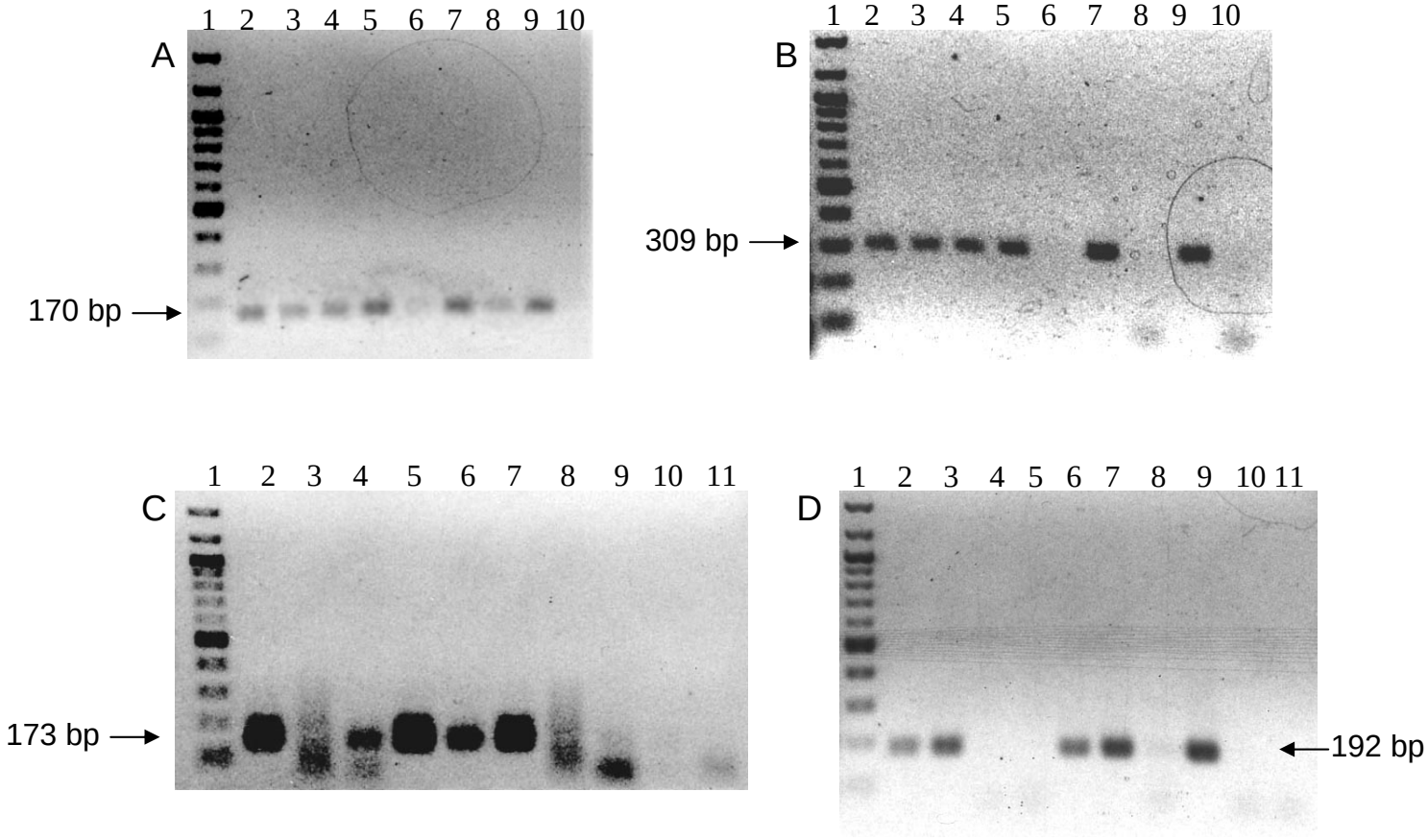
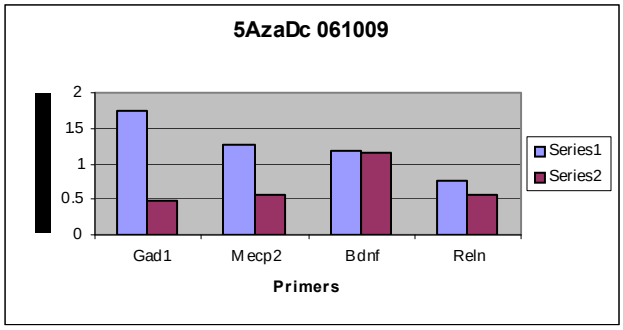


Fig. 3. PCR products obtained with bisulfite sequencing (BS) or Methylation Specific (MS, NMS) primers were analyzed by agarose gel electrophoresis. In lane 1 of each gel: 100 bp DNA ladder. Bisulfite-Treated DNA was prepared from Astrocytes (A) or Neurons (N) using DNA samples from cells lysed either with Dneasy (lanes 2,3, 4, and 7) or Trizol reagent (lanes 5, 6, 7, and 9). Astrocytes DNA: lanes 2, 3 and 4 (Dneasy); lanes 5, 6 and 7 (Trizol). Neurons DNA: lane 8 (Dneasy) and lane 9 (Trizol). A: PCR with Mecp2 MS primers 7M2F/8M2R (Table3). B: PCR with BS Bdnf primers F32/R4 (Table 1). C: PCR with NMS Reln primers 30F/17R (Table 2). D: PCR with MS Gad67 primers F16/R13 (Table2).

A



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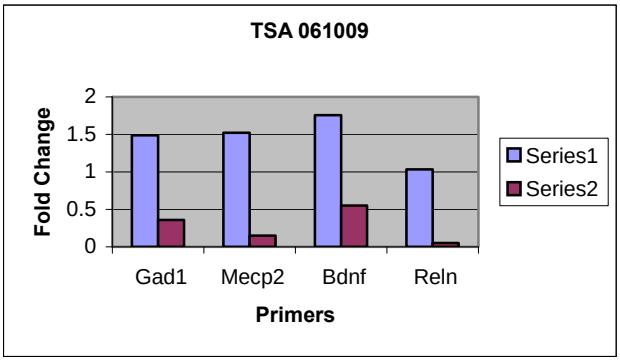

C

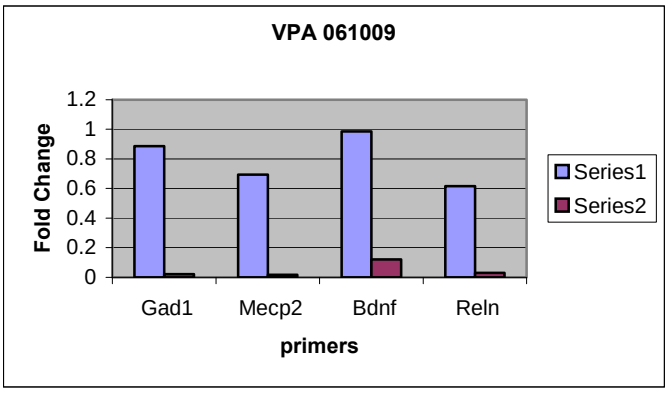

Fig. 4. Effects of treatment with 5AzadC (A), TSA (B) and VPA (C) of primary neurons on gene expression. mRNA was extracted, converted to cDNA and used in Real Time PCR (Table 4). Fold change between treated and untreated cells was calculated using the $2^{-\Delta\Delta C}{}_{T}$ Method using Hprt mRNA expression as reference. The standard deviation is shown in red.